J Pharmacol Sci **105**, 122 – 126 (2007)

Journal of Pharmacological Sciences

Short Communication

Nobiletin, a Citrus Flavonoid That Improves Memory Impairment, Rescues Bulbectomy-Induced Cholinergic Neurodegeneration in Mice

Akira Nakajima[1,#], Tohru Yamakuni[1,#], Mitsuya Haraguchi[1], Naoki Omae[1], Si-Young Song[2], Chieko Kato[2], Osamu Nakagawasai[3], Takeshi Tadano[3], Akihito Yokosuka[4], Yoshihiro Mimaki[4], Yutaka Sashida[4], and Yasushi Ohizumi[1,5,6,*]

[1]*Department of Pharmaceutical Molecular Biology, Graduate School of Pharmaceutical Sciences, Tohoku University, Aoba, Aramaki, Aoba-ku, Sendai 980-8578, Japan*
[2]*Institute of Neuroscience, Tokushima Bunri University at Kagawa, 1314-1 Shido, Sanuki-shi, Kagawa 769-2193, Japan*
[3]*Department of Pharmacology, Tohoku Pharmaceutical University, 4-4-1 Komatsushima, Aoba-ku, Sendai 981-8558, Japan*
[4]*Laboratory of Medicinal Plant Science, School of Pharmacy, Tokyo University of Pharmacy and Life Science, Hachioji, Tokyo 192-0392, Japan*
[5]*Yokohama College of Pharmacy, 601 Matano-cho, Tozuka-ku, Yokohama 245-0066, Japan*
[6]*Research Center of Supercritical Fluid Technology, Graduate School of Engineering, Tohoku University, 6-6-11-404 Aoba, Aramaki, Aoba-ku, Sendai 980-8579, Japan*

Received March 24, 2007; Accepted June 21, 2007

Abstract. We have recently reported that nobiletin, a citrus flavonoid, improves impaired memory in olfactory-bulbectomized (OBX) mice, which have been widely utilized as a useful paradigm that shares some major clinical features of Alzheimer's disease. Here, we examined the effects of nobiletin on OBX-induced cholinergic neurodegeneration in mice. OBX mice showed reduced acetylcholinesterase (AChE) staining and choline acetyltransferase (ChAT) expression in the hippocampus. An 11-day administration of nobiletin rescued OBX-induced decrease in the density of AChE-staining and ChAT expression in the hippocampus. These results suggest that nobiletin rescues OBX-induced cholinergic neurodegeneration, accompanied by improvement of impaired memory in OBX mice.

Keywords: Alzheimer's disease, cholinergic neuron, nobiletin

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by cognitive and memory deterioration, with a devastating impact on the whole society. During recent decades there has been a dramatically growing awareness about the urgency of seeking more effective therapeutic interventions for patients with AD. The basal forebrain cholinergic neurons play a critical role in learning and memory. In patients with AD, cholinergic neuronal loss is particularly noticeable in the neocortex and hippocampus (1). Such a loss of basal forebrain cholinergic neurons underlies the behavioral and cognitive deficits observed in AD (1).

Olfactory-bulbectomy (OBX) results in a retrograde degeneration of the neurons that project to and from the main and accessory olfactory bulbs (2). OBX animals have been widely utilized as a useful paradigm that shares some major clinical features of AD, since the animal model exhibits impaired learning and memory caused by degeneration of the CNS cholinergic system (3, 4). A decrease in acetylcholine contents in rat brain after OBX was also reported (5).

Large numbers of compounds from natural resources have provided not only useful pharmacological tools but also novel leading compounds for drug development. In the course of our survey of substances having anti-AD drug activity from natural resources, we successfully found nobiletin, a flavonoid from *Citrus depressa* (Fig. 1), as a candidate for a novel type of anti-AD drug

[#]Present address: Division of Pharmacotherapy, Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai 980-8578, Japan
*Corresponding author (Affiliation #6).
ohizumi@scf.che.tohoku.ac.jp
Published online in J-STAGE
doi: 10.1254/jphs.SC0070155

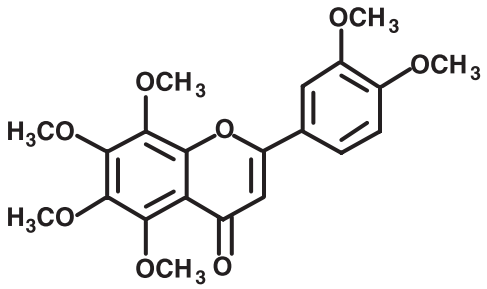

Fig. 1. Chemical structure of nobiletin.

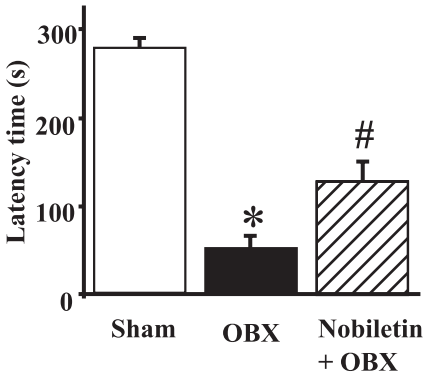

Fig. 2. Effects of a daily administration of nobiletin for 11 days on OBX-induced memory impairment in the passive avoidance task. The training session was performed before OBX. Mice were administered nobiletin (50 mg/kg, i.p.) or vehicle (0.5% Tween 80) for 11 days (from the 3rd day to the 13th day after OBX). The retention test was performed on the 14th day after OBX. Data are means ± S.E.M. (n = 24 – 28). *P<0.01 *vs* sham. $^{\#}P$<0.01 *vs* OBX.

with a unique mechanism of action. Nobiletin triggers an activation of cAMP/PKA/ERK/cAMP-responsive element binding protein (CREB) signaling and improves impaired memory in OBX mice (6, 7). Here, we provide the evidence that nobiletin rescues OBX-induced cholinergic neurodegeneration in the hippocampus.

Adult male ddY mice (24.5 ± 1.5 g) were obtained from Nippon SLC (Hamamatsu). Animals were housed in cages with free access to food and water under the condition of constant temperature (23 ± 1°C) and humidity (55 ± 5%) and adapted to a standard 12-h light / 12-h dark cycle (light cycle: 9:00 – 21:00). The procedures used in this study were approved by the Committee on the Care and Use of Experimental Animals, Tohoku University in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health. The OBX mice were prepared as described previously (4). Briefly, mice anesthetized with sodium pentobarbital (50 mg/kg, i.p.; Dainippon, Osaka) were placed on a stereotaxic instrument. The olfactory bulbs (OB) were bilaterally aspirated by a suction pump after the scalp was incised, the openings drilled over the bulbs, and the dura cut. Sham-operated mice were treated in the same way, but the bulbs were left intact.

The training trial and retention test of the passive avoidance task were conducted as described previously (7). When the mouse completely entered the dark compartment, an electric shock (1 mA for 500 ms) was supplied to the floor bars. Mice were exposed to the electric shock before surgery. Nobiletin at 50 mg/kg per day or vehicle (0.5% Tween 80) was intraperitoneally given to OBX mice consecutively for 11 days from day 3 after OBX. The dose of nobiletin was chosen on the basis of our previous study showing that nobiletin dose-dependently rescues β-amyloid peptide (Aβ)-induced impairment of learning and memory (8). Each mouse was placed in the light compartment and step-through latency was recorded until 300 s had elapsed (retention test) on day 14 after OBX. After completion of the retention test, mice were subjected to acetylcholinesterase (AChE)-staining or Western blot analysis of choline acetyltransferase (ChAT). Animals used for histological analysis were given an overdose of sodium pentobarbital (70 mg/kg) and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.2). Frozen sections were cut at 30 μm using a cryostat and AChE-staining was conducted by the free-floating method following the procedure described previously (9). The quantification of the intensity of AChE-staining was assessed by NIH image. The locations in which the measurements were made are shown in Fig. 3B. Animals used for western blot analysis were killed by cervical dislocation, and the hippocampus was dissected out on an ice-cold glass plate. Western blotting was performed as described previously (7). Goat anti-ChAT antibody (Chemicon International, Inc., Temecula, CA, USA) and rabbit anti-14-3-3 β antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used to detect ChAT and 14-3-3 β, respectively. Statistical analyses were made using one-way ANOVA followed by Fisher's PLSD posthoc tests. A level of P<0.05 was considered statistically significant.

We used the passive avoidance task in which animals learn to associate a location with an aversive stimulus. This task has been previously shown to be hippocampal-dependent (10). The step-through latency of the retention test in OBX mice was shown to be markedly decreased on day 14 after the operation (Fig. 2). Daily administration of 50 mg/kg of nobiletin for 11 days from day 3 after OBX resulted in an increase in the step-through latency on day 14 (Fig. 2). These results suggest that nobiletin improves impaired memory in OBX mice.

The AChE-staining experiment showed that OBX

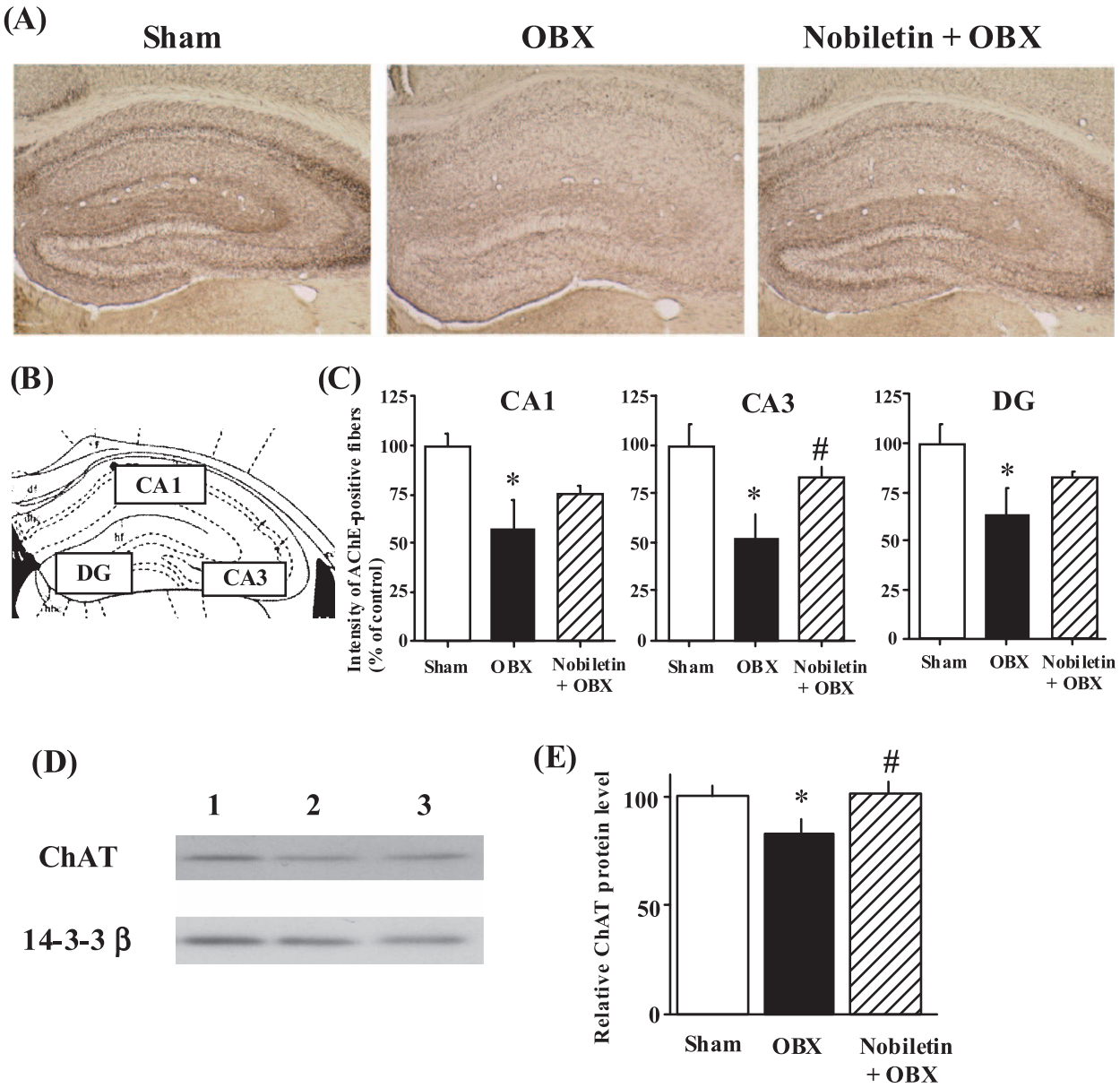

Fig. 3. Effects of nobiletin on OBX-induced decrease in the density of AChE-positive fibers and ChAT expression in the hippocampus. A: Representative photomicrographs of AChE-stained sections in the hippocampus of sham-operated (left), OBX (middle), and nobiletin-treated OBX mice (right). B: The locations in which the intensity of AChE-staining was measured are illustrated in the diagram of coronal sections. The drawing is based on the atlas of Franklin and Paxinos (15). C: Quantitative analysis of the intensity of AChE-staining in the hippocampus. Data are means ± S.E.M. (n = 5). *P<0.05 *vs* sham. $^{\#}P$<0.05 *vs* OBX. D: Representative western blots of ChAT levels of sham (lane 1), OBX (lane 2), and nobiletin-treated OBX mice (lane 3). E: Densitometric analysis of ChAT levels. Following probing with anti-ChAT antibody, blots were reprobed with anti-14-3-3 β antibody to verify that equal amount of proteins were loaded on each lane. Data are means ± S.E.M. (n = 9 – 10). *P<0.05 *vs* sham. $^{\#}P$<0.05 *vs* OBX.

reduced the staining in the hippocampus (Fig. 3A), but in contrast, rarely affected the staining in the cortex (data not shown). The density of AChE-staining in the CA1, CA3, and dentate gyrus (DG) of the hippocampus in OBX mice were reduced by 37% – 48% in comparison to that of sham-operated mice ($F_{2,12} = 4.8972$, P<0.05; $F_{2,12} = 6.1643$, P<0.05; $F_{2,12} = 3.9495$, P<0.05; respectively; Fig. 3C). An 11-day intraperitoneal administration of nobiletin rescued the OBX-induced decrease in the density of AChE-positive fibers in the hippocampus by 19 – 32% (Fig. 3). An ANOVA followed by the PLSD test revealed a significant difference in AChE-staining between OBX and nobiletin-treated OBX mice in the CA3 region (P<0.05). In addition, there is a

tendency to recover the density of AChE-staining in the CA1 and DG, although the difference was not statistically significant. Furthermore, Western blot analysis showed that the treatment with nobiletin also rescued the OBX-induced decrease in ChAT expression in the hippocampus (Fig. 3: D and E). These results suggest that nobiletin rescues OBX-induced cholinergic neurodegeneration in the hippocampus.

It has been reported that in the hippocampus, the intensity of AChE-staining reflects the density of cholinergic septohippocampal innervation (11) and that behavioral improvement is correlated with recovery of cholinergic markers such as AChE and ChAT (12). Previous studies showed that memory deficits in OBX mice were improved by stimulation of the cholinergic system (4). The antidepressant minaprine, which increased the release of acetylcholine in the hippocampus by blocking the presynaptic 5-HT_2 receptor on the cholinergic neurons (13), similarly improved the memory impairment in OBX animals (3). Taken together, it is plausible that nobiletin improves impaired memory in OBX mice by protecting the cholinergic innervation in the hippocampus of OBX mice. Antidepressive effects of nobiletin in OBX animals should be examined in future studies. In addition, it should be noted that nobiletin significantly improves memory impairment and to a greater extent, the decreased level of AChE activity in OBX mice. The difference in the extent of the improving effects of nobiletin may be in part attributable to changes in endocrine, immune, and neurotransmitter systems other than the cholinergic system in OBX animals (2). Further studies are needed to examine the effects of nobiletin on OBX-induced changes in these systems.

What is the mechanism by which nobiletin protects the cholinergic innervation in the hippocampus of OBX mice? We have recently reported that nobiletin activates an extracellular signal-regulated kinase (ERK) / mitogen-activated protein kinase (MAPK)-dependent signaling pathway to trigger CREB phosphorylation and CRE-dependent transcription in cultured hippocampal neurons and PC12D cells (6, 7). In addition, nobiletin reverses learning impairment associated with *N*-methyl-D-aspartate (NMDA)-receptor antagonism by activation of ERK signaling in the hippocampus of mice (14). A number of studies have shown that the CREB/CRE-dependent transcription pathway regulates the expression of genes involved in synapse formation, neuronal survival (BDNF, BCl-2), and long-term memory formation (C/EBP) (10). These observations lead us to the hypothesis that nobiletin induces protection of cholinergic nerve fibers in the hippocampus of OBX mice by activation of the ERK/CREB signaling pathway to trigger gene expression involved in neuroprotection.

In addition, we have recently found that repeated treatment with nobiletin (50 mg/kg, for 7 days) enhanced contextual fear memory when mice were trained with a single foot shock, although no memory enhancement was found after extensive training (H. Onozuka et al., unpublished observation). Also, we have found that nobiletin treatment stimulates ChAT mRNA expression in PC12D cells (S. Hamaoka et al., unpublished observation). Nobiletin reverses sublethal concentration of Aβ_{1-42}-induced reduction in the activity of PKA /CREB-dependent signaling pathway in cultured rat hippocampal neurons (8). This compound also ameliorates Aβ-induced impairment of memory in AD model rats (8). Collectively, nobiletin could be a candidate for an anti-AD drug with a novel mechanism of action.

References

1 Bartus RT. On neurodegenerative diseases, models, and treatment strategies: lessons learned and lessons forgotten a generation following the cholinergic hypothesis. Exp Neurol. 2000; 163:495–529.

2 Song C, Leonard BE. The olfactory bulbectomised rat as a model of depression. Neurosci Biobehav Rev. 2005;29:627–647.

3 Yamamoto T, Jin J, Watanabe S. Characteristics of memory dysfunction in olfactory bulbectomized rats and the effects of cholinergic drugs. Behav Brain Res. 1997;83:57–62.

4 Hozumi S, Nakagawasai O, Tan-No K, Niijima F, Yamadera F, Murata A, et al. Characteristics of changes in cholinergic function and impairment of learning and memory-related behavior induced by olfactory bulbectomy. Behav Brain Res. 2003;138:9–15.

5 Yoshimura H, Gomita Y, Ueki S. Changes in acetylcholine content in rat brain after bilateral olfactory bulbectomy in relation to mouse-killing behavior. Pharmacol Biochem Behav. 1974;2:703–705.

6 Nagase H, Yamakuni T, Matsuzaki K, Maruyama Y, Kasahara J, Hinohara Y, et al. Mechanism of neurotrophic action of nobiletin in PC12D cells. Biochemistry. 2005a;44:13683–13691.

7 Nagase H, Omae N, Omori A, Nakagawasai O, Tadano T, Yokosuka A, et al. Nobiletin and its related flavonoids with CRE-mediated transcription-stimulating and neuritegenic activities. Biochem Biophys Res Commun. 2005b;337:1330–1336.

8 Matsuzaki K, Yamakuni T, Hashimoto M, Haque AM, Shido O, Mimaki Y, et al. Nobiletin restoring β-amyloid-impaired CREB phosphorylation rescues memory deterioration in Alzheimer's disease model rats. Neurosci Lett. 2006;400:230–234.

9 Hedreen JC, Bacon SJ, Price DL. A modified histochemical technique to visualize acetylcholinesterase-containing axons. J Histochem Cytochem. 1985;33:134–140.

10 Taubenfeld SM, Milekic MH, Monti B, Alberini CM. The consolidation of new but not reactivated memory requires hippocampal C/EBPbeta. Nat Neurosci. 2001;4:813–818.

11 Heckers S, Ohtake T, Wiley RG, Lappi DA, Geula C, Mesulam

MM. Complete and selective cholinergic denervation of rat neocortex and hippocampus but not amygdala by an immunotoxin against the p75 NGF receptor. J Neurosci. 1994;14:1271–1289.

12 Tarricone BJ, Simon JR, Low WC. Intrahippocampal transplants of septal cholinergic neurons: Choline acetyltransferase activity, muscarinic receptor binding, and spatial memory function. Brain Res. 1993;632:41–47.

13 Muramatsu M, Tamaki-Ohashi J, Usuki C, Araki H, Aihara H. Serotonin-2 receptor-mediated regulation of release of acetylcholine by minaprine in cholinergic nerve terminal of hippocampus of rat. Neuropharmacology. 1988;27;603–609.

14 Nakajima A, Yamakuni T, Matsuzaki K, Nakata N, Onozuka H, Yokosuka A, et al. Nobiletin, a citrus flavonoid, reverses learning impairment associated with NMDA receptor antagonism by activation of ERK signaling. J Pharmacol Exp Ther. 2007;321:784–790.

15 Franklin KBJ, Paxinos G. The mouse brain in stereotaxic coordinates. San Diego: Academic Press; 1997.